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Light and auxin treatments affect morphogenesis and polyphenolics productivity in *Artemisia alba* Turra cell aggregates in vitro

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Abstract
Artemisia alba Turra is an essential oil-bearing shrub, with a Euro-Mediterranean distribution widespread in the south-eastern parts of Europe. Phytochemical investigations have evidenced the presence of volatile mono- and sesquiterpene derivatives, as well as non-volatile sesquiterpenoids, flavonoids and phenolic acids contributing to the anti-inflammatory, antimicrobial, antioxidant and pro-apoptotic activity of different preparations, obtained from the plant. The current research aims at elucidation of the potential for biotechnological polyphenolic compounds productivity of non-differentiated cell lines of the plant. For this purpose, non-differentiated cell aggregates were initiated from either leaf or root explants of the sterile grown plant. They were cultivated either in the dark or at 16/8 h photoperiod in liquid media, supplemented with N^6-benzyladenine (BA) as auxin. The cytokinin effects of indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) were compared. It was established that NAA supplementation was superior to IBA and light treatment – to dark growth conditions in terms of polyphenolics productivity. In addition, NAA supplementation led to better expressed compaction and larger size of the cell aggregates as compared with IBA. The results of the present experiment indicate that secondary metabolites productivity *in vitro* is a dynamic process closely related to the plant's growth and development and is in close relation to the interactions of the plant with its environmental conditions.

Keywords
Artemisia alba Turra cell suspensions, auxins, cytokinins, polyphenolics productivity in vitro

Introduction

The production, accumulation and translocation of secondary metabolites within the integral plant organism are determined by the presence and organisation of highly specialised anatomical structures. Thus, growth, development and morphogenesis of the plant individual play crucial roles for the production and accumulation of secondary metabolites. Since growth and development are dynamically related to the interactions of the plants with their surrounding environment, the latter plays a crucial role in determining the plants secondary metabolite profile. Plant cell tissue and organ culture represents the convenience of a controlled environment with the flexibility to alter selected factors related to *in vitro* cultivation and, thus, modify the production of desired secondary metabolites *in vitro* (Danova 2018).

Genus *Artemisia*, tribe Anthemideae, subtribe Artemisiinae is one of the largest in the Asteraceae family with more than 500 species and subspecies (Zhen et al. 2010). Representatives of the genus have been included in traditional medicinal preparations from centuries for treatment of conditions such as fever, high blood pressure, diabetes, gastrointestinal disorders, parasites etc. (Tu 2016; Zeb et al. 2018; Danova 2020). Scientific evidence for the pharmacological potential of *Artemisia* species is the presence of triterpenes, steroids, hydrocarbons, polyacetylenes, flavonoids, coumarins, mono- and sesquiterpenoids isolated from representatives of the genus (Maggio et al. 2012). Research has confirmed the cytotoxic, antihepatotoxic, anti-bacterial, antifungal and antioxidant properties of preparations of the species (Tan et al. 1998; Bora and Sharma 2011).

A. alba Turra (syn. *A. lobelii* All, *A. camphorata* Vill., *Artemisia biasolettiana* Vis, *A. suavis* Jord., *A. incanescens* Jord.) is an essential oil-bearing shrub natively distributed in south-eastern Europe (Radulović and Blagojević 2010; Biondi and Galdenzi 2012). Its decoction has been traditionally used in the Mediterranean Region as a tonic and stomach digestive (Rigat et al. 2007). Phytochemical studies on the volatile (camphor, 1,8–cineole and artemisia ketone dominating components of the essential oil) and non-volatile (kaempferol, luteolin and apigenin derivatives, rutin, oxygenated sesquiterpenoids, chlorogenic acid, dicaffeoylquinic acids, scopoletin, umbeliferone etc.) constituents of the species have been performed, giving the scientific grounds for the established anti-microbial, anti-inflammatory, antioxidant properties of the species (Stojanovic et al. 2000; Stalińska et al. 2005; Danova et al. 2020).

In previous research conducted on differentiated shoot cultures of the species, it was established that the development of the root system as a result of exogenous plant growth regulators treatment was decisive for the profile of the essential oils, derived from the aerial parts of *A. alba* Turra. Thus, plantlets with well-developed root system (in plant growth regulators-free media, as well as in media supplemented with 0.5 mg/l or 1.0 mg/l indole-3-butyric acid (IBA)) were characterised with over 2.5 times higher ratio of the mono/sesquiterpenoids content in the essential oil, as compared with plantlets with supressed rooting and callus formation at the explant base (in media where 0.5 mg/l or 1.0 mg/l IBA were combined with 0.2 mg/l benzyl adenine (BA)) (Danova et al. 2018).

Continuing the previous research on *Artemisia alba*'s biotechnological properties, we set as an aim to develop a fast-growing non-differentiated *in vitro* system of *Artemisia alba* Turra with high biosynthetic potential regarding the production of polyphenolics. A comparative analysis of the morphogenic and biosynthetic response of liquid cell-aggregate cultures derived from leaf and root explants with different photoperiod and different type of auxin (IBA or 1-Naphthylacetic acid, NAA), in the presence of the same cytokinin (BA) was conducted.

Materials and methods

Plant material

Shoot cultures were initiated through surface sterilisation of stem explants of field grown *A. alba* as previously described (Danova et al. 2012). Stock shoot cultures were kept on plant growth regulators (PGR) – free medium supplemented with the Murashige and Skoog (Murashige and Skoog 1962) macro- and micro-salts medium. Stock cultures were kept at a temperature of 25 ± 0.1 °C, 16/8 h photoperiod, light intensity 60 µmol $m^{-2}s^{-1}$, period of sub-cultivation 4 months.

Tissue culture experiment

For the needs of this research, leaf (A1) and root (A2) explants of the *in vitro* grown stock plants were used. Five leaf (A1) and root (A2) explants were inoculated into two types of liquid media with total volume 50 ml in Erlenmeyer flasks (Table 1). Media were prepared with macro- and micro-elements and vitamins according to Murashige and Skoog (1962), 30 $g.l^{-1}$ sucrose and the cytokinin benzyladenine. We experimented with two types of auxin – indole-3-butyric acid and naphthylacetic acid as shown in Table 1. For each treatment, five separate culture vessels were placed in two types of light conditions – 16/8 photoperiod and in the dark. All flasks were placed on an orbital shaker, 100 rpm. That led to the formation of the eight experimental lines (Table 1). The biological experiment of non-differentiated cell aggregate culture induction from explants of the sterile grown plan was repeated in triplicate.

Table 1. The eight *A. alba* experimental variants.

		BA [$mg.l^{-1}$]	IBA [$mg.l^{-1}$]	NAA [$mg.l^{-1}$]	Photoperiod
1	A1 ER_3 hv	0.1	1.5	-	16/8
2	A2 ER_3 hv	0.1	1.5	-	16/8
3	A1 ER_3NAA hv	0.1	-	1.5	16/8
4	A2 ER_3NAA hv	0.1	-	1.5	16/8
5	A1 ER_3	0.1	1.5	-	Dark
6	A2 ER_3	0.1	1.5	-	Dark
7	A1 ER_3NAA	0.1	-	1.5	Dark
8	A2 ER_3NAA	0.1	-	1.5	Dark

Morphometric observations

The dynamics of the morphometric response of explants was tracked by stereomicroscopy using the Stereomicroscope Leica M60. Stereomicroscopy was performed 2 and 4 months after the initial induction of the cell lines. For the observations, plant material from at least two separate culture vessels was sampled.

Biochemical analyses

The quantitative content of malondialdehyde (Dhindsa et al. 1981) and H_2O_2 (Jessup et al. 1994) were assayed spectrophotometrically.

Phytochemical analyses

Spectrophotometric determination of the total content of phenolic (Singleton et al. 1999) and flavonoid compounds (Zhishen et al. 1999) was performed. For the analyses, plant material from at least four separate culture vessels was sampled. Measurements were performed in triplicate.

Statistical processing

The respective number of samples tested, measurements and biological repetitions have been mentioned at each method. The SEM values (standard error of the mean) are reflected in Figures. The means have been compared by t-test of unequal variances at $P < 0.05$. Unless otherwise stated, differences are considered statistically significant at $P \leq 0.05$.

Results and discussion

Impact of growth regulators and photoperiod on growth and development

Changes in the fresh/dry weight ratio, two and four months after induction of the suspension lines are shown in Figure 1. Biomass accumulation was influenced both by the type of initial explant (leaf or root) and the photoperiod. When comparing this parameter 2 and 4 months after initiation, the different capacity of dry biomass accumulation (expressed by a lower FW/DW ratio) in the different treatments is more noticeable. Two months after the initial induction, the FW/DW ratio of the eight lines has relatively similar values, the variants grown in the light tending to be more hydrated (expressed by a higher FW/DW ratio), compared to those grown in the dark (except A2 ER_3NAA).

In addition, a higher water accumulation tendency was observed in lines obtained from root (A2) vs. the ones obtained from leaf explant (A1). Two months after the in-

duction, no cell lines, being strongly productive in terms of dry biomass accumulation, could be observed, the FW/DW parameter being relatively similar in-between them.

However, after two consecutive passages of the suspension cultures (4 months after induction), cell lines grown in the light showed a significant drop in the FW/DW ratio, indicating the tendency of higher dry biomass accumulation.

Some of the cell lines grown in the dark (A1 ER_3, A1 ER_3NAA and A2 ER_3NAA) show a higher result compared to the ones grown in the light, indicating higher hydration, as the difference in leaf explants treated with IBA (A1 ER_3) is the most significant. This may be due to both reduced abilities to accumulate dry biomass and differences in the density of cell aggregates in cultures and the change in osmotic pressure in cells. The largest differences in the FW/DW ratio are observed in leaf explants grown in the dark A1 ER_3 and A1 ER_3NAA, which could be explained by the impaired photosynthetic ability due to lack of light (Fig. 1).

Stereomicroscopy of the eight plant lines 2 months after induction.

When performing the stereomicroscopy 2 months after induction, the presence of differentiated plant organs and tissues was still observed (Fig. 2). It was more pronounced in root (A2) vs. stem (A1) explants and in NAA treatments as compared with IBA ones. In the light-grown lines, intense green pigmentation was clearly observed, as compared with the ones grown in the dark, including both light-grown root lines, which is a visual indication of the formation of photosynthetic pigments. In the case of leaf explants with both auxins, callus formation was observed and, in the variant treated with IBA in light, it was less dense, lighter in colour and presence of antho-

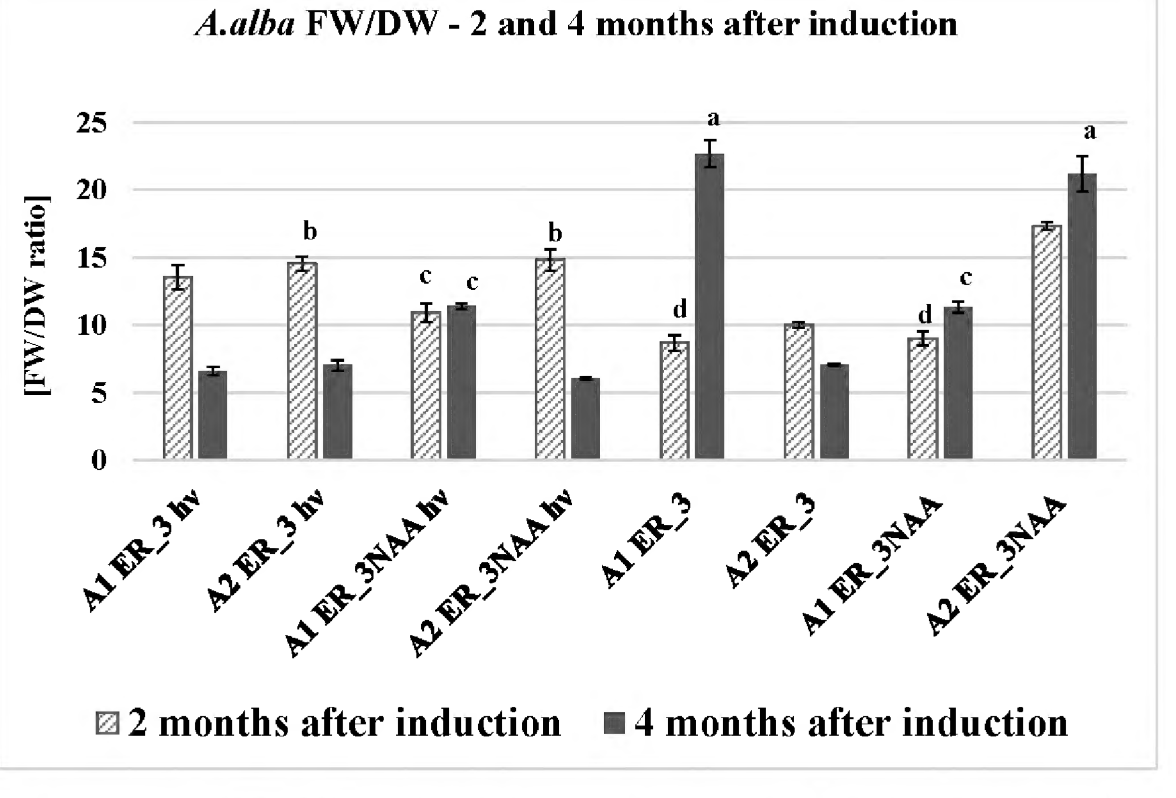

Figure 1. Changes in the FW/DW ratio 2 and 4 months after initial induction of the cell lines, respectively. Same letters denote statistically non-significant differences.

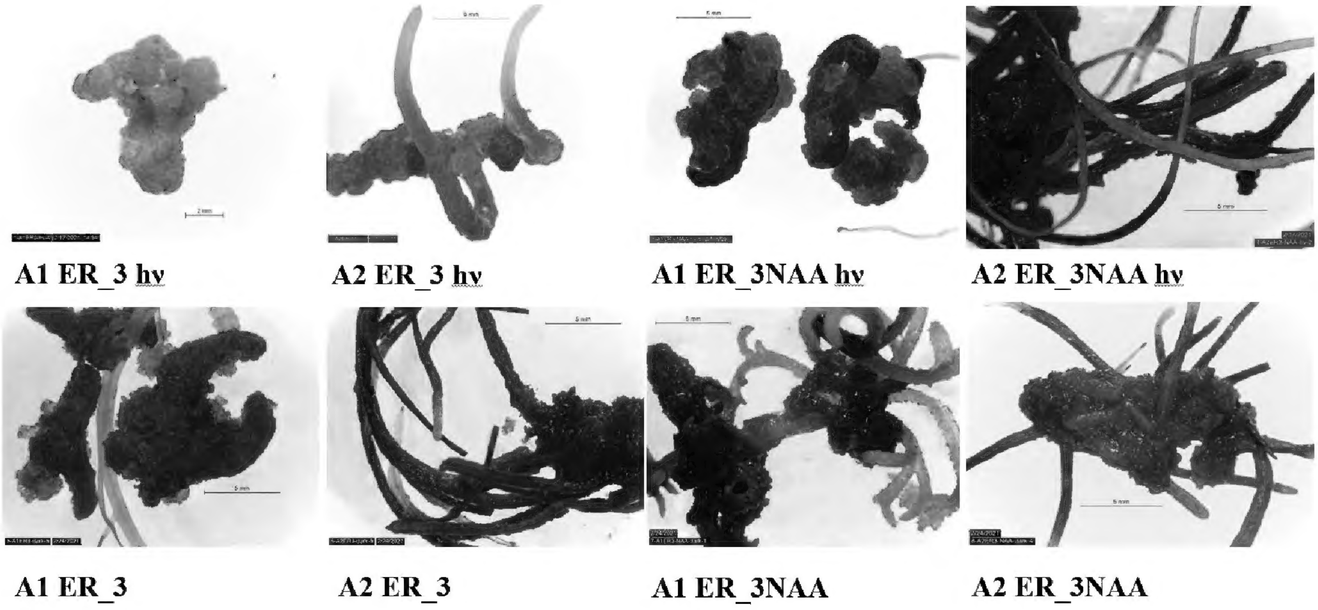

Figure 2. Stereomicroscopic imaging of the eight plant lines 2 months after initial line induction.

cyanin pigments was observed, in contrast to the variant treated with NAA, in which we observed intense green colouration, denser cell aggregates and relatively stronger organogenesis. The tendency for stronger organogenesis under the influence of NAA was maintained in the dark, as the leaf explant clearly showed many etiolated (due to lack of light) stems.

Stereomicroscopy of the eight cell lines 4 months after induction

When performing the same analysis 4 months after the initial induction (after two passages of the induced structures described earlier), the degree of differentiation decreased significantly, as indirect organogenesis was again observed more clearly in the lines obtained from root explants (A2) (Fig. 3). There were no significant differences in terms of indirect organogenesis when comparing the NAA and IBA treatments.

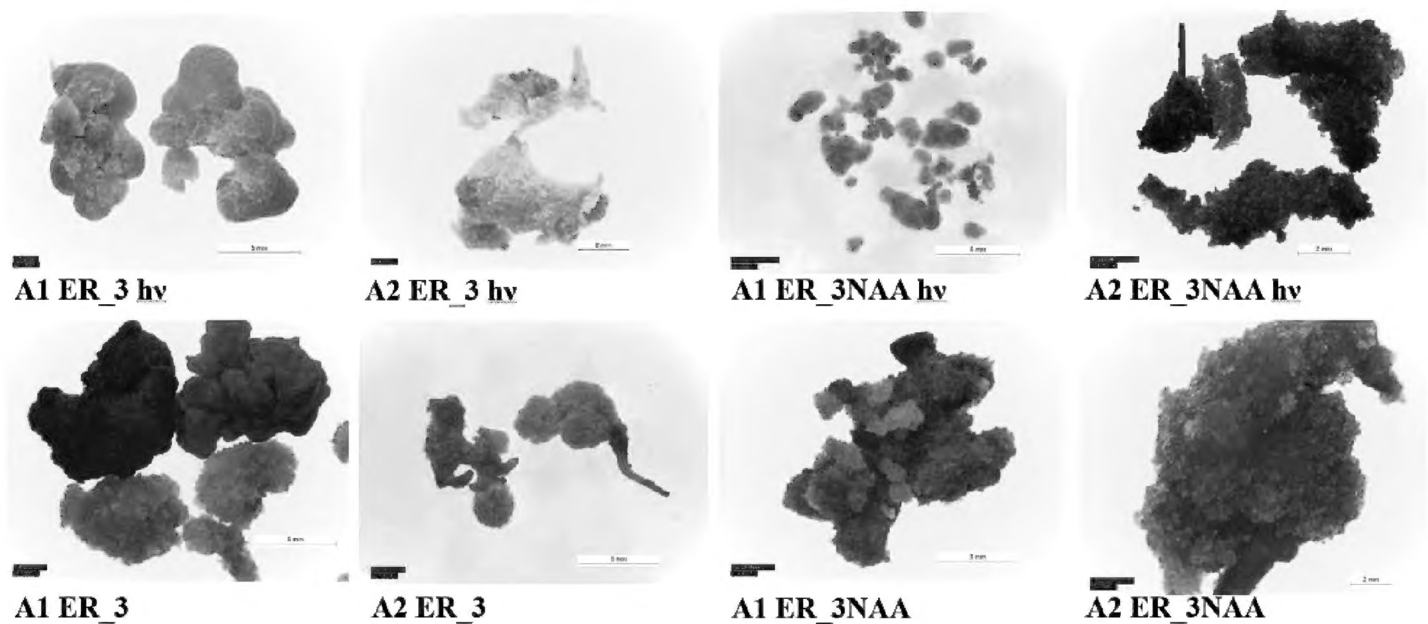

Figure 3. Stereomicroscopic imaging of the eight plant lines 4 months after initial line induction.

IBA-treated lines formed larger cell aggregates, which, however, were less dense, more brittle and looser. The cell aggregates obtained by NAA treatment were smaller in size, but significantly denser and more compact. In the case of aggregates, obtained from leaf explants in the light, we can assume the presence of photosynthesis to some extent, due to the preservation of the intense green colour. After stabilisation, the A2 lines, both the light and dark, formed aggregates with white to brownish colour, without green colouration.

Photographic characterszation of the eight cell lines 6 months after the initial induction

Six months after the initial induction and after 3 passages, all cultures were already completely de-differentiated (Fig. 4). The formation of cell aggregates was also observed in the eight cell lines and they were relatively larger in size in the lines induced by leaf (A1), as compared with the ones obtained from the root (A2) explant. In the lines obtained from leaf explant grown in the dark (A1 ER_3, A1 ER_3NAA), there was a gradual loss of colour in the de-differentiated cultures. A weak green staining was only preserved in the lines obtained from light-grown leaf explants (A1 ER_3 hv, A1 ER_3NAA hv), being weaker in the NAA treatment. Sporadic morphogenesis was only observed in the A2 lines, in support of the assumption that they were more difficult to bring to a fully de-differentiated culture.

MDA content in the eight experimental lines

Changes in malondialdehyde content in the eight plant lines are shown in Figure 5. In both light and dark, MDA is relatively high in leaf explants compared to root and the trend is more visible in variants grown in the presence of light.

Again, this may be due to the more active photosynthesis under illumination and the presence of a larger relative amount of photosynthetic tissue in the original stem

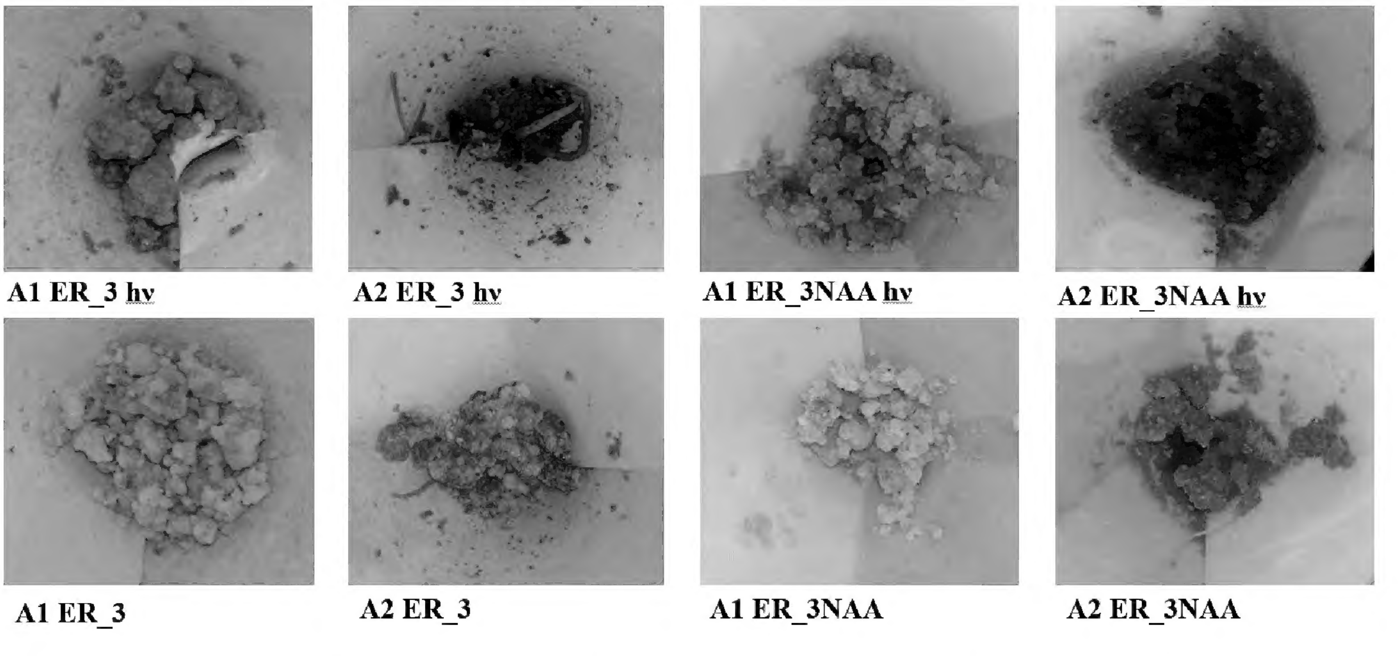

Figure 4. Photographic characterisation of the eight plant lines 4 months after initial line induction.

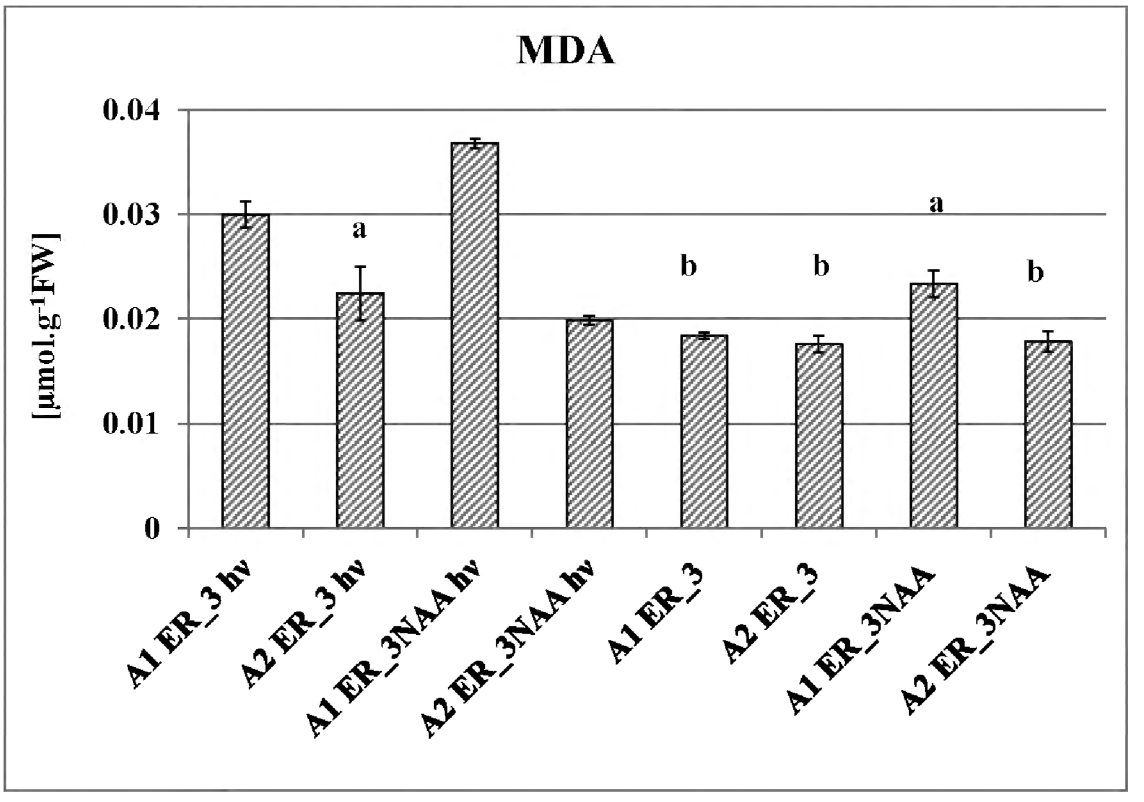

Figure 5. Changes in malondialdehyde (µmol.g^{-1} FW) content in lines, induced from leaf and root of *A. alba* under the influence of different growth regulators and at different photoperiods. $p < 0.05$. Statistically insignificant differences in the average values of the measured parameters are indicated in identical letters.

explant. The drop of MDA levels in the absence of light may be due to inhibition of photosynthetic processes in A1 lines, respectively generation of less active forms of oxygen and lower lipid peroxidation. We can note that the trend observed in MDA was also maintained in the amount of H_2O_2 – light-grown variants, expressing significantly higher values than in the dark-grown ones, which can again be explained by the decrease in photosynthetic activity.

Hydrogen peroxide content in the eight experimental lines

Changes in hydrogen peroxide content in the eight plant lines are shown in Figure 6.

There was a slight decrease in the amount of hydrogen peroxide in the root explants treated with NAA compared to those treated with IBA.

The relations with the type of explant and the auxin applied was also clear – when using NAA, A2 lines always had lower levels of H_2O_2, respectively lower levels of oxidative stress, while, when using IBA, there was an inverse dependency – always A2 lines had higher levels of H_2O_2, regardless of the light treatment. When comparing the two parameters, we can note, as a tendency, that both MDA and H_2O_2 were higher in light-grown lines, probably due to the generation of ROS from the ongoing photosynthetic processes. However, in light-derived A2 lines explants, the lower MDA as compared to A1 lines did not correlate with higher H_2O_2 levels, indicating that, in the presence of light, the root tissue generated high H_2O_2 levels, which led to activation of the antioxidant protection of the cells and a correspondingly lower degree of lipid oxidation. In the case of lines obtained from leaf explants grown in the light, both parameters were high

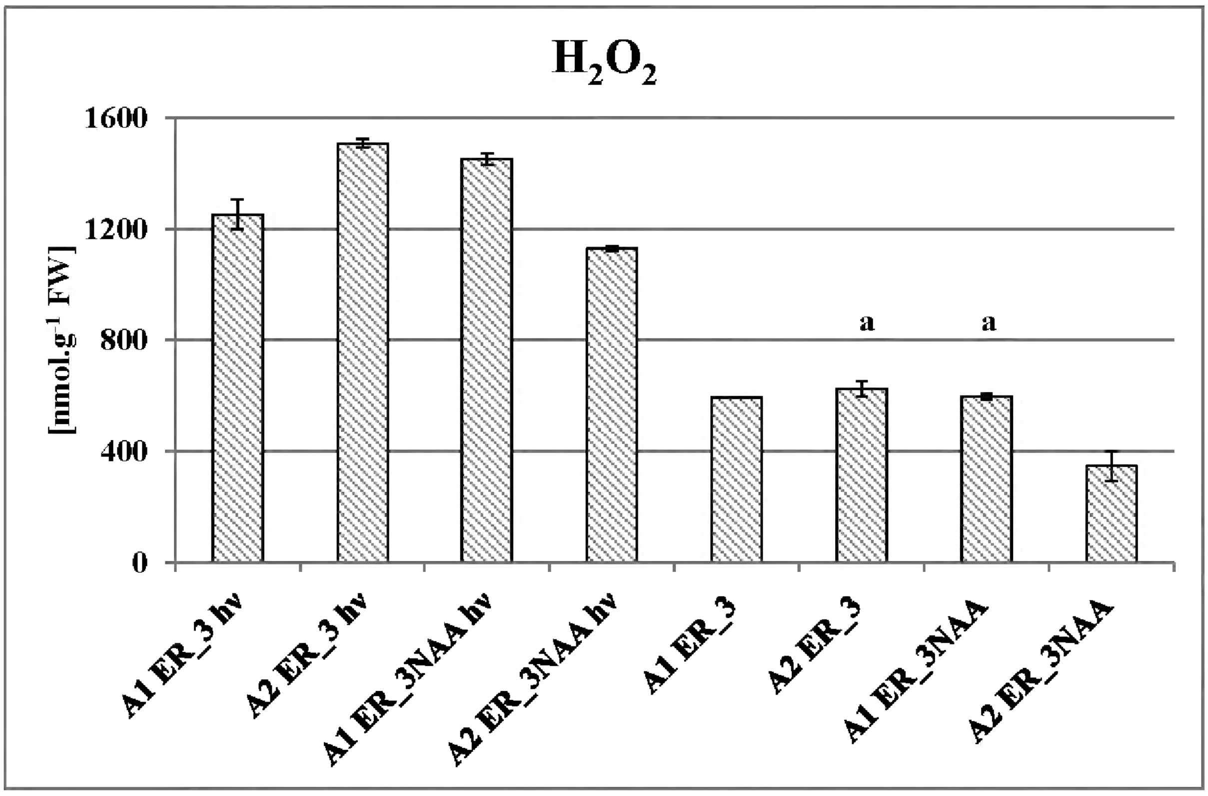

Figure 6. Changes in hydrogen peroxide (nmol.g^{-1} FW) content in lines, induced from leaf and root of *A. alba* under the influence of different growth regulators and at different photoperiods. $p < 0.05$. Statistically insignificant differences in the average values of the measured parameters are indicated in identical letters

and, in the case of lines obtained from leaf explants grown in the dark, they dropped. Probably the A1 lines, grown in the light, retain their photosynthetic ability to some extent and, accordingly, this leads to the generation of both MDA and H_2O_2, while, in the dark, this ability is limited and we see that, both morphologically, they completely lose their green pigmentation, together with the significant drop of MDA and H_2O_2, indicating lower levels of stress, less ROS and a lower degree of lipid peroxidation.

Content of phenols and flavonoids in the eight experimental lines

Phenolic and flavonoid compounds content in the eight plant lines is presented in Figure 7 and Figure 8, respectively.

Phenolic compounds are part of the plant's cellular response to various types of stress. Accordingly, we can assume that their content would increase with a source of stress, such as light.

The experiments show a dependence with a similar trend – the variants grown in the 16/8 photoperiod produced higher amounts of phenolic compounds than those grown in the dark (Fig. 7). There was also a tendency for the amount of phenolic compounds to be significantly higher in the root explants obtained lines, as compared to the leaf ones. Their amount was significantly higher (58% and 140%) in the root-derived lines treated with NAA, as compared to the root-derived explants treated with IBA. The trend that we observed in the levels of total phenolic compounds was analogical as far as flavonoids were concerned (Fig. 8). Again, we observed higher flavonoid levels in the light-grown lines, as compared to the dark-grown ones, which can be

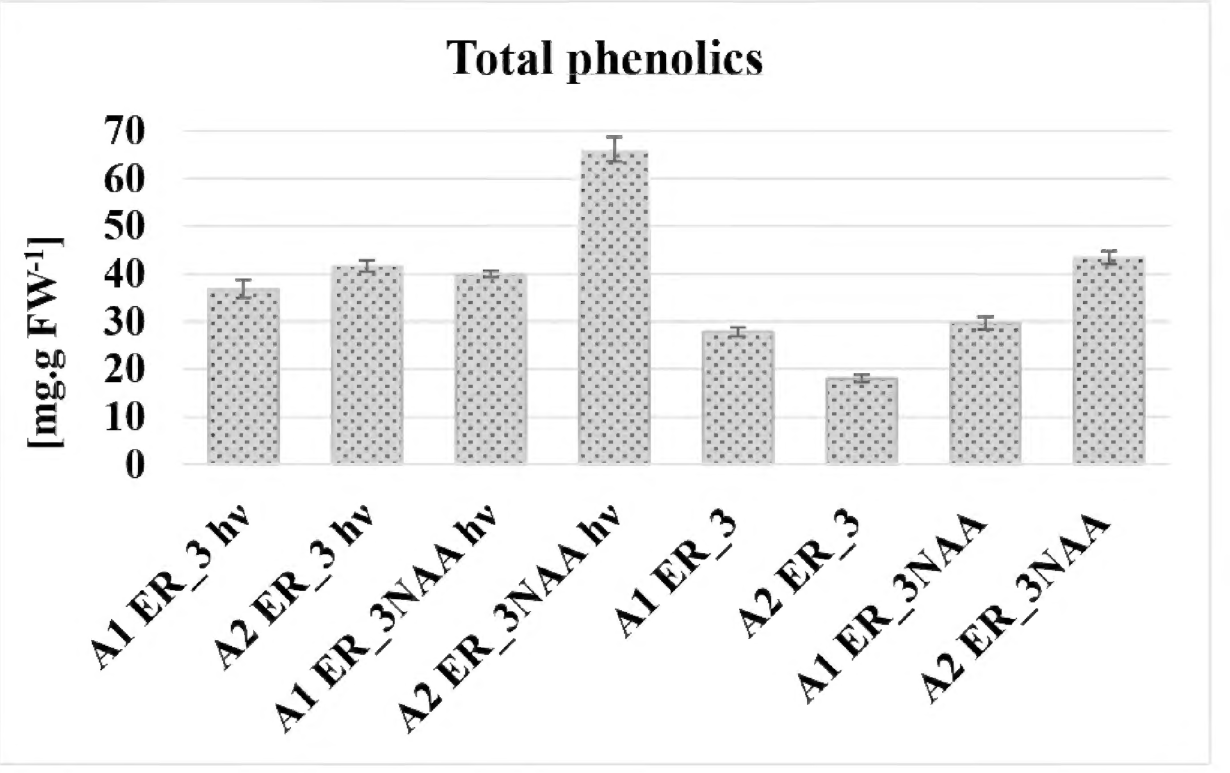

Figure 7. Phenolics content (mg. g^{-1} FW) in lines induced from leaf and root explants of *A. alba* under the influence of different growth regulators and at different photoperiods.

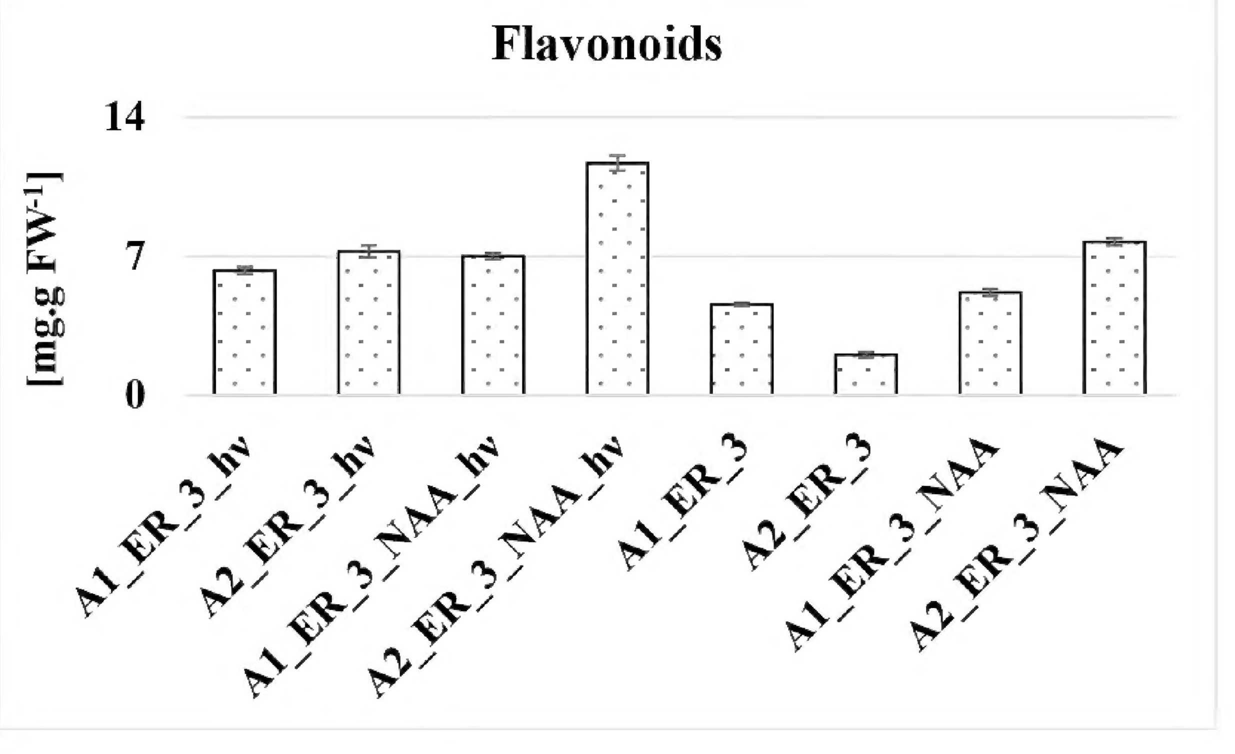

Figure 8. Flavonoid content (mg. g^{-1} FW) in lines induced from leaf and root explants of *A. alba* under the influence of different growth regulators and at different photoperiods.

explained by the higher photosynthetic activity. Thus, the observations on the degree of de-differentiation and its correlation with the levels of MDA, H_2O_2 and phenolic compounds showed that, in the lines initiated by root explant (A2), de-differentiation was much slower, a higher degree of tissue hyperhydricity in the early stages of development was observed and scarce root morphogenesis still occurred even after several consecutive passages. Nevertheless, root-derived cell aggregates of *A. alba* (with the

exception of A2_ER3) were shown to possess the highest biosynthetic capacity of phenolic and flavonoid compounds. Flavonoids have a wide range of applications in pharmacy, well known pharmacological properties, for example, activation or inhibition of specific enzymes, incl. cyclo-oxygenase, lipoxygenase, detoxification of carcinogens and other proven health benefits for humans and animals (Pollastro et al. 2018). They are thought to be involved in controlling the growth and differentiation of plant cells and tissues. Accordingly, higher levels of phenolic and flavonoid compounds of those lines may be related to their impaired ability to de-differentiate. Phenolic compounds have a high absorption capacity; however, evidence for their direct involvement in photosynthetic processes is not yet known (Agati et al. 2012).

Root explants treated with NAA and grown in the light (A2_ER3 hv) showed the best values of all four studied parameters (lipid peroxidation, oxidative stress, phenolic and flavonoid productivity). They were characterised by a strong hydration after the initial induction of the culture, which, however, decreased significantly after the second passage. Accordingly, this line could be distinguished as relatively more productive in this respect.

Artemisia alba Turra *in vitro* was shown to respond to the introduction in a de-differentiated state by activating both biochemical and physiological mechanisms, which were characterised by great dynamics.

The aim of this experiment was to establish the most competent cell lines in terms of biosynthesis of phenolics and flavonoids, experimenting with the presence or absence of light, the type of plant tissue used for induction of cell line (stem and root explants) and with the type of auxin added – IBA and NAA. Regarding the formation of cell aggregates and the differentiation of cultures, clear differences were observed according to the type of auxin used. Lines treated with IBA were easier to de-differentiate, forming bulky cell aggregates, which were, however, less dense. NAA-treated lines show more difficult de-differentiation, especially in those derived from root explants (A2), but after de-differentiation, they formed cell aggregates relatively smaller in size, denser and with a lower fresh/dry weight ratio, respectively, with better biomass accumulation.

In terms of stress markers, again light-grown cell lines showed elevated values compared to those grown in the dark, with the levels of MDA being significantly higher in leaf explants (A1), showing high levels of lipid peroxidation.

In terms of the amount of phenolic and flavonoid compounds, the root lines had a significantly higher synthetic capacity compared to those obtained from leaf explants. There was also a clear dependence on the type of auxin used and, again, NAA shows better results.

Conclusion

When we take into consideration everything said above, we can conclude that, despite the slower and more difficult de-differentiation, in terms of the synthesis of phenolic and flavonoid compounds, the most favourable parameters have been established for the lines obtained from root explants, when NAA was applied as auxin. A2 ER_3NAA_hv and A2 ER_3NAA stand out as the most productive lines for the

production of the target compounds. This may provide future guidance for the development of high-yielding root lines for the synthesis of secondary metabolites from the studied plant *Artemisia alba* Turra.

Acknowledgements

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